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Cadherin-mediated cell adhesion and tissue segregation: qualitative and quantitative determinants

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Abstract

It is widely held that segregation of tissues expressing different cadherins results from cadherin-subtype-specific binding specificities. This belief is based largely upon assays in which cells expressing different cadherin subtypes aggregate separately when shaken in suspension. In various combinations of L cells expressing NCAM, E-, P-, N-, R-, or B-cadherin, coaggregation occurred when shear forces were low or absent but could be selectively inhibited by high shear forces. Cells expressing P- vs E-cadherin coaggregated and then demixed, one population enveloping the other completely. To distinguish whether this demixing was due to differences in cadherin affinities or expression levels, the latter were varied systematically. Cells expressing either cadherin at a lower level became the enveloping layer, as predicted by the Differential Adhesion Hypothesis. However, when cadherin expression levels were equalized, cells expressing P- vs E-cadherin remained intermixed. In this combination, "homocadherin" (E-E; P-P) and "heterocadherin" (E-P) adhesions must therefore be of similar strength. Cells expressing R- vs B-cadherin coaggregated but demixed to produce configurations of incomplete envelopment. This signifies that R- to B-cadherin adhesions must be weaker than either "homocadherin" adhesion. Together, cadherin quantity and affinity control tissue segregation and assembly through specification of the relative intensities of mature cell–cell adhesions.

Keywords: Cadherin; Cell adhesion; Cell affinity; Cell sorting; Differential adhesion; Homophilic; Morphogenesis; Segregation; Sorting-out; Specificity

Introduction

Embryonic development is marked by the segregation of embryonic germ layers and then of their incipient tissues, which migrate over one another's surfaces and the extracellular matrix as they rearrange to take up their characteristic positions in the body plan. Dramatic examples of these rearrangements are seen during gastrulation, neurulation, and organogenesis. In early studies of the causes of these tissue rearrangements, it was found that normal tissue layering could be achieved in vitro (Holtfreter, 1939), not only by the normal process of mutual tissue spreading but also by the sorting-out of experimentally intermixed cells within coherent multicellular aggregates (Holtfreter, 1944; Townes and Holtfreter, 1955; reviewed in Armstrong, 1989). Moreover, organ-like structures with specific anatomical configurations could be formed in like manner by combinations of cells or tissues that normally never encounter each other in the embryo (Moscona, 1957; Trinkaus and Groves, 1955). Analyses of the behavior of cells and tissues as they carried out these in vitro rearrangements led to the Differential Adhesion Hypothesis, which proposes that these rearrangements result from the repeated exchange of weaker for stronger adhesions by intrinsically motile cells. The final configuration, approaching that of minimal interfacial free energy, is achieved when total cell–cell binding strength is maximized (Steinberg, 1962a–c, 1963, 1964, 1970). Important elements of this explanation have been confirmed by studies of the behavior of heterogeneous combinations of cells and tissues, by computer-modeling (Glazier and Graner, 1993; Goel and Leith, 1970; Goel et al., 1970; Graner, 1993; Graner and Sawada, 1993; Mostow, 1975; Palsson, 2001; Palsson and Othmer, 2000) and by direct

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physical measurements (Davis et al., 1997; Foty et al., 1994, 1996). To assess the roles of particular cell adhesion molecules in tissue segregation, in vitro studies of the behavior of cell populations selected or engineered to express identified adhesion molecules in measured amounts offer powerful tools.

The cadherins are a superfamily of transmembrane Ca^{2+}-dependent cell–cell adhesion molecules (Kemler, 1992), within which the "classical" cadherins constitute a highly homologous subgroup. The "type I" classical cadherins (Suzuki et al., 1991) are comprised of a series of molecular subtypes, e.g., E-cadherin (-cad), P-cad, etc. Cadherin-mediated cell–cell adhesions are "homophilic" in the sense that cadherins represent both the "locks" and the "keys" on apposed cell surfaces. The importance of cadherins in morphogenetic processes was first suggested by the observation that cell rearrangements during development are often associated with changes in cadherin subtype (reviewed in Takeichi, 1988). Major morphological defects occurred when cadherin function was blocked with antibodies (Bronner-Fraser et al., 1992; Matsunaga et al., 1988) and when cadherins were ectopically expressed (Detrick et al., 1990; Fujimori et al., 1990), in a series of in vivo experiments. Those findings demonstrate that proper cadherin expression and function are required for normal tissue segregation. In light of the importance of the cadherins in cell–cell adhesion, a question central to understanding their dynamic role in embryogenesis concerns cadherin selectivity. To what extent can the different members of the type I classical cadherin subfamily engage in heterocadherin bonding?

A much-cited conviction has held that cadherin-mediated cell–cell bonding is cadherin-type-selective, reflecting a fundamental disaffinity between different cadherin subtypes. This opinion has arisen largely from reports that mixed suspensions of dispersed cells transfected to express different members or subtypes of the "classical" cadherin family (Kemler, 1992), when stirred together, in some instances reaggregate separately in a largely cadherin subtype-specific manner. Cadherin subtype adhesive specificity is also widely invoked to explain the segregation, within a contiguous tissue mass, of embryonic tissues and the demixing ("sorting-out") of intermixed populations of cohering cells expressing different cadherins (e.g., Takeichi, 1988, 1990). However, in the case of cell and tissue segregation mediated entirely by cadherins, it has been shown that purely quantitative differences in expression levels of a single cadherin suffice to produce these rearrangements in the absence of any difference in cadherin subtypes. Such quantitative differences are sufficient to automatically specify both the sorting-out of intermixed cells (Friedlander et al., 1989; Steinberg and Takeichi, 1994) and which cell population, of an apposed pair, will envelop the other (Steinberg and Takeichi, 1994). However, there has been no equivalent converse demonstration of the ability of differences in cadherin subtype to produce these behaviors in the absence of differences in cadherin expression levels. The present experiments were undertaken to reexamine the ability of cells uniquely expressing different cadherin subtypes to cross-adhere and to evaluate the relative contributions made to tissue segregation behavior by differences in cadherin subtype binding specificities vs differences in cadherin expression levels.

Materials and methods

Antibodies

PCD-1 and NCD-2 (Zymed Laboratories, South San Francisco, CA) were used for P-cad- and N-cad-expressing cells, respectively. ECCD-1 and ECCD-2 (Zymed) were used for E-cad-expressing cells. Hybridoma lines expressing mouse antibodies specific for N-cad (6B3; George-Weinstein et al., 1997), B-cad (5A6; Murphy-Erdosh et al., 1994), and R-cad (MRCD-2; Redies et al., 1992) were gifts from K. Knudsen, L. Reichardt, and M. Takeichi, respectively. W. Gallin supplied us with a rabbit polyclonal antibody against LCAM.

Cell lines

All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (fcs), 50 units/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin, and 10 μg/ml gentamicin in a humidified 5% CO_2 atmosphere. By calcium phosphate coprecipitation (Chen and Okayama, 1987), L929 cells (American Type Culture Collection, Rockville, MD) were transfected with pBATEM2, pβact-Pcad (Nose et al., 1988), and pMiwcN (Fujimori et al., 1990), expression vectors for murine E-, murine P-, and chicken N-cad, respectively. The cells were then selected in complete medium containing G418 at 400 μg/ml active geneticin. The selection medium was changed every 3 days to remove cell debris and supply fresh G418. After about 2 weeks of selection, single colonies were isolated by using cloning rings (Freshney, 1983) and expanded. Clones were screened by flow cytometry for reactivity to antibodies specific for E-cad (ECCD-2; Shirayoshi et al., 1986), P-cad (PCD-1; Nose and Takeichi, 1986), or N-cad (NCD-2; Hatta and Takeichi, 1986). Positive cells were then subcloned. The N-cad-expressing lines were autocloned into 96-well plates by using the CloneCyt Integrated Deposition System (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Positive clones were reanalyzed by flow cytometry. The N-cad lines designated N2 and N5A, expressing different measured levels of N-cad, the E-cad line designated E8a, and the P-cad line designated LP1 were used here. An L cell line (LE-Dex) expressing E-cad under the control of a glucocorticoid-inducible promoter (pLK-neo; Nose and Takeichi, 1986) was obtained from W. James Nelson (Angres et al., 1996).

Maximal expression of E-cad in these cells was achieved by incubating the cells overnight in complete medium containing up to 1 μM dexamethasone. An L cell line expressing R-cad (Inuzuka et al., 1991) was obtained from M. Takeichi. L cell lines expressing LCAM and B-cad (Murphy-Erdosh et al., 1995) were obtained from L. Reichardt.

Cell labeling

Cells were stained with either PKH26 red fluorescent cell marker or PKH67 green fluorescent cell marker, according to the instructions of the manufacturer (Sigma Chemical Co., St. Louis, MO). Briefly, 5×10^6 dissociated cells were washed in phosphate-buffered saline, then resuspended in 200 μl of the appropriate diluent. The cell suspension was then added to 200 μl of 10^{-5} M dye in the same diluent. The cells were incubated for 2 min at 25°C, with regular agitation to assure thorough mixing. Then, 1 ml of serum was added to stop the reaction, and the cells were then washed three times in 10% fcs/DMEM. We have discerned no effect of this staining procedure upon the behavior of the cells.

Cell dissociation and aggregation

Subconfluent plates of cadherin-transfected L cells were treated with either 0.05% trypsin/0.53 mM EDTA in Ca^{2+}- and Mg^{2+}-free HBSS (TE treatment, for all aggregation experiments) or 0.05% trypsin in HBSS with 2 mM $CaCl_2$ (TC treatment, for measurement of cadherin expression levels) until they were released from the plate. The cells were then labeled to fluoresce red or green as described above and resuspended in 10% fcs/DMEM at 10^6 cells of each type/ml. For aggregation under laminar flow, 1 ml of cell suspension was transferred to a screw-capped 4-ml flat-bottomed Sample Vial (Wheaton Science Products, Millville, NJ) and rotated horizontally around the vial's axis at a constant rate of between 1 and 40 rpm at 37°C. For aggregation under gyration, cell suspensions in 10% fcs/DMEM were transferred to 10-ml round-bottomed flasks (Bellco, Vineland, NJ), 3 ml per flask, and gyrated in a water-bath shaker (New Brunswick Scientific, Model G76) at 37°C, 5% CO_2. In cases where function-blocking antibodies were used, they were included in the culture medium at 100 μg/ml. When EGTA was used to chelate the Ca^{2+} present in the medium, it was included at 3–5 mM. Cell aggregates were fixed in 3.7% formaldehyde.

Sorting-out (demixing) of cells within aggregates

Cells were dissociated by TE treatment, labeled as described above, and placed in gyratory culture (see above) for 2 h at 37°C, 120 gyres/min to allow the cells to recover from trypsin treatment. A total of 10^6 cells of each of the two cell lines to be combined was transferred to a screw-capped round-bottomed glass tube 15 mm in diameter and gently centrifuged to form a thin pellet. The tube was incubated overnight to allow the pellet to tighten. The resulting pellets were then cut into fragments about 1 mm square and incubated in 10-ml tissue culture flasks on the water-bath shaker for 48 h, during which time the multicellular fragments rounded up to become spheroids. These were fixed in 3.7% formaldehyde and viewed by confocal microscopy as described below.

Quantification of cadherin expression

For this application, cells were dissociated by TC treatment. Ca^{2+} present in the medium protects the exposed cadherin molecules from digestion by trypsin (Takeichi, 1977).

Relative E-cad expression levels on uninduced vs induced LE-Dex cells were determined by flow cytometric analysis. Following dissociation, cells were washed in 10 ml HBSS, then resuspended in 100 μl of a solution containing a saturating concentration of 10 μg/ml of rat monoclonal antibody ECCD-1. The cells were incubated on ice with gentle mixing for 60 min, then washed with 10 ml HBSS. The cells were resuspended in an Alexa 488 goat anti-rat IgG conjugate (Molecular Probes, Eugene, OR) at 10 μg/ml in HBSS, incubated 60 min on ice, and then washed with 10 ml HBSS. They were then resuspended in 1 ml HBSS and analyzed by using a FACScan Analyzer [Becton-Dickinson Immunocytometry Systems (BDIS), San Jose, CA] and Cell Quest software (BDIS). For each analysis, 10,000 gated events were collected.

Absolute cadherin expression levels were determined for N-cad-, P-cad-, and certain E-cad-expressing lines [E8a, LE-Dex (uninduced)] by a quantitative flow cytometric assay (Brockhoff et al., 1994; Zagursky et al., 1995) using Quantum Simply Cellular (QSC) microbeads (Flow Cytometry Standards Inc., San Juan, PR) following the manufacturer's protocol. The QSC kit contains five populations of microbeads, a blank (negative control) and four Simply Cellular microbead populations, which display different calibrated binding capacities for mouse or rat IgG monoclonal antibodies. For N-cad-expressing cells, we employed Fab fragments of the 6B3 N- cadherin antibody generated using the Immunopure Fab Preparation Kit (Pierce) following the manufacturer's instructions. Two milligrams of Fab were coupled to NHS Sulfo Biotin (Pierce). Biotinylated Fab fragments were further purified by FPLC. QSC microbeads and transfected cells were incubated in 20 μg/ml biotinylated 6B3 Fab for 1 h at 4°C, washed several times, then resuspended in 10 μg/ml streptavidin–phycoerythrin. Rat mAbs ECCD-1 and PCD-1 were used for E- and P-cad-expressing cells, respectively, followed by goat anti-rat IgG coupled to Alexa 488 fluorochrome. After several washes, beads and cells were analyzed by using the FACScan flow cytometer and software supplied with the QSC beads.

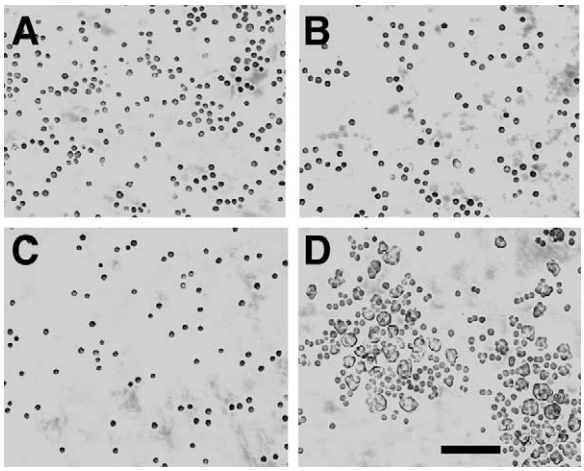


Fig. 1. Cadherin-dependent L cell aggregation. Untransfected L cells dissociated by treatment with trypsin–EDTA (A) and cultured in suspension on a gyratory shaker at 60 gpm for 4.5 h (B) did not aggregate. N5A cells, expressing N-cadherin (Table 1), dissociated in the same manner (C) aggregated significantly during this time (D). Phase-contrast images. Scale bar represents 100 μm.

Confocal microscopy and imaging

Confocal images of the green and red fluorescence were obtained with a BioRad MRC600 scanning laser confocal microscope system attached to a Nikon Optiphot-2 microscope and saved as separate files by using CoMOS software, version 7.0a (BioRad Microscience Division, Cambridge, MA). The two optical channels were then merged and assigned the proper colors by using the Confocal Assistant Software, version 2.55.

Results

These studies focus on the contributions of cadherin identity, cadherin abundance, and fluid mechanics to two kinds of adhesion-related cell population behavior. These are (1) the tendency of a pair of cell populations to aggregate either separately or together in a stirred suspension and (2) the tendency of a pair of cell populations that aggregate together to remain intermixed thereafter or to demix (sort out) into separate domains within the common aggregates. The results are grouped below in the stated sequence.

Aggregation of cadherin-expressing L cell populations

When dissociated L cells, which lack cadherins, were cultured in a stirred suspension for many hours, they failed to aggregate (Figs. 1A and 1B). (This was true even in very gently sheared cell suspensions. They associate in very loose clusters only when the cells are maintained in stationary cultures on a substratum to which they adhere very poorly; Ryan and M.S.S., unpublished observations). L cells transfected to express functional cadherin molecules aggregated very well under the same conditions during this time (Figs. 1C and 1D). When the cells were dissociated with trypsin–EDTA, which degrades surface cadherins, the aggregation began in about 20 min, as surface cadherins were restored. If, however, the cells were dissociated with trypsin-Ca^{2+}, which protects surface cadherins, the cells began aggregating immediately.

Cross-adhesion between different cadherins

To determine whether different type I cadherins expressed on the surfaces of apposed cells can form adhesive cross-links ("heterocadherin" adhesions) in sheared cell suspensions, we combined transfected L cells expressing different type I cadherins in our laminar flow roller tube device under slow rotation in order to increase cell–cell contact times and decrease adhesion-disrupting shear forces. Laminar flow also permits estimation of the shear forces applied.

R-cad and B-cad

Two TE-suspended L cell populations, expressing B-cad (green) or R-cad (red), rotated together in the laminar flow device at the extremely low rate of 1.2 rpm for 90 min, coaggregated indiscriminately (Fig. 2A).

LCAM and N-cad

TE-dissociated L cells expressing N-cad (red) were mixed in the laminar flow device with others expressing LCAM (green), the putative chicken homolog of mammalian E-cad. At 3.3 rpm, indiscriminate association was evident after 2 h of aggregation (Fig. 2B).

P-cad and E-cad

TE-suspended L cells expressing E-cad (green) or P-cad (red) were mixed in equal numbers and maintained at 15 rpm in the laminar flow device for 100 min. As noted above, a combination of L cells expressing these two cadherins has previously been reported to aggregate separately in shaken suspensions, giving rise to the belief that these two cadherins do not cross-adhere. Under the conditions used here, however, these cell populations coaggregated indiscriminately (Fig. 2C).

B-cad and N-cad

N-cad-expressing cells (red) and B-cad-expressing cells (green), rotated together at 6 rpm for 70 min in the laminar flow device, also coaggregated indiscriminately (Fig. 2D).

Inhibition of heterocadherin adhesion by specific antibodies and Fabs

Aggregation of all of the above cadherin-transfected L cell lines required the presence of Ca^{2+}, a characteristic of cadherin-mediated cell aggregation. The above observations led us to conclude that the coaggregation of cells in all of the combinations cited was mediated by heterocadherin

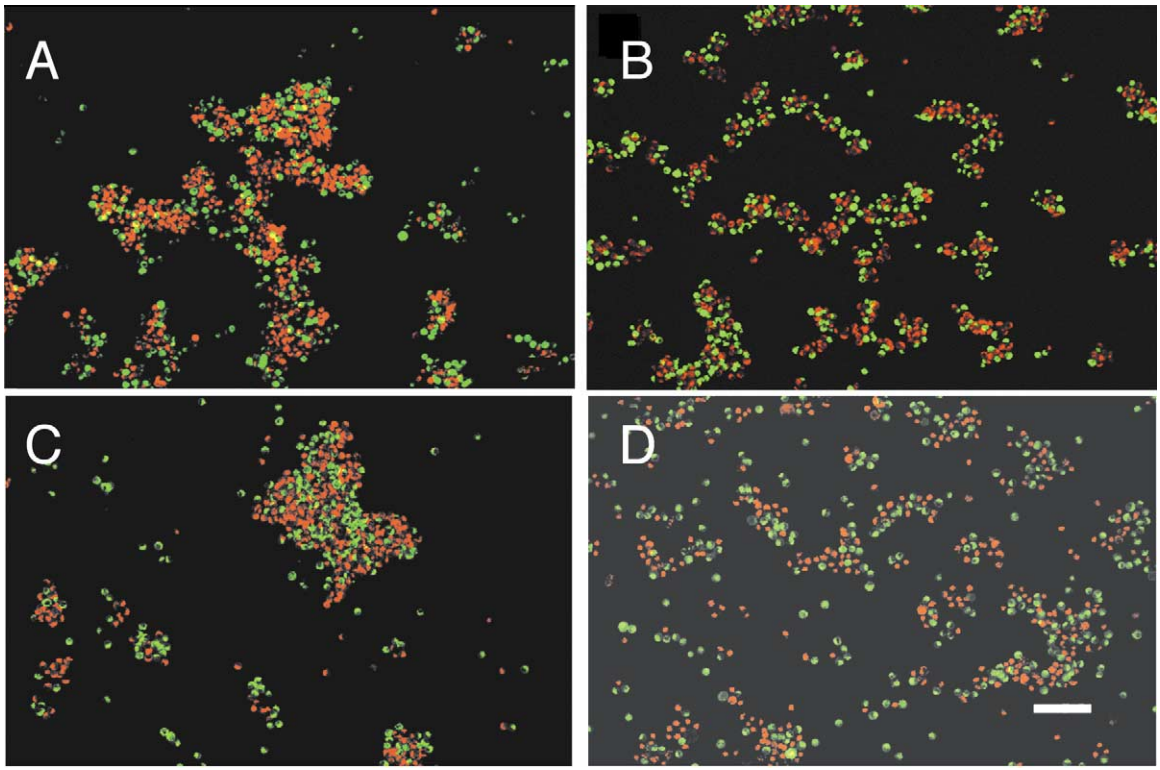


Fig. 2. Cross-adhesion between cells expressing different type I cadherins. L cells expressing B-cad (green) vs R-cad (red) coaggregated during 90 min at 1.2 rpm (A). L cells expressing LCAM (green) vs N-cad (red) coaggregated within 2 h at 3.3 rpm (B). L cells expressing P-cad (red) vs E-cad (green) coaggregated under all shear conditions tested, shown in (C) after 100 min at 15 rpm in the laminar flow device. L cells expressing N-cad (N2, red) vs B-cad (green) coaggregated in the laminar flow device during 70 min at 6 rpm (D). All aggregations were carried out at 10^6 cells of each type/ml. Differences in the number of cells per photographic field are due to cell aggregation and sampling. Confocal images. Scale bar represents 100 μm.

interactions. As an independent test of this conclusion, we have examined the capacity of cadherin-specific inhibitory antibodies and Fabs to inhibit the formation of these adhesions.

P-cad and E-cad

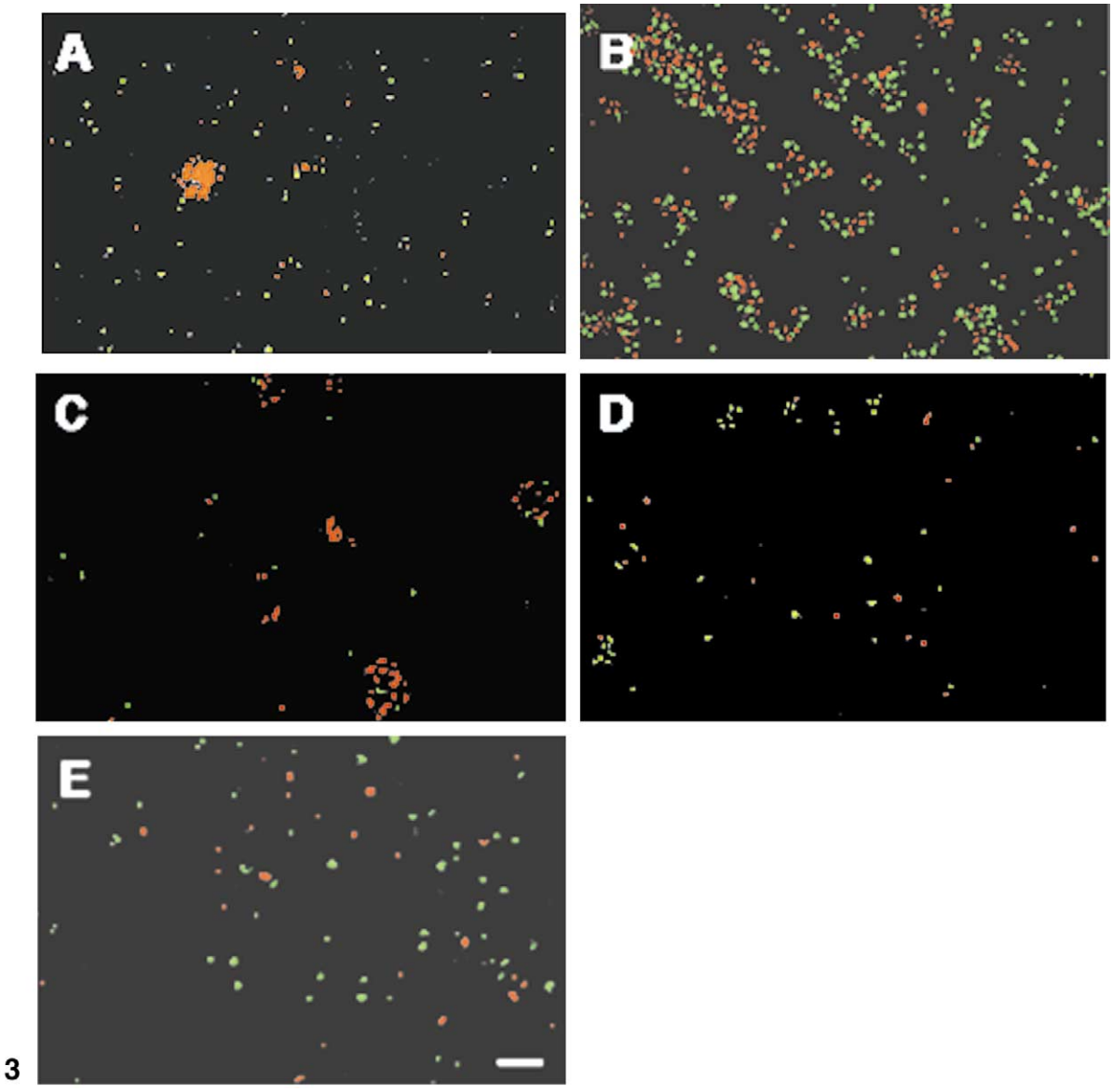
As has been noted above, mixtures of E- and P-cadherin-expressing cells coaggregated. In the presence of 100 μg/ml ECCD-1, a function-blocking E-cad antibody (Ogou et al., 1983), the P-cad- expressing cells (red) proceeded to aggregate, but the E-cad-expressing cells (green) adhered in significant numbers neither to each other nor to the P-cad-expressing cells, even under relatively low shear forces (Fig. 3A). Under the same circumstances, ECCD-2, a non-function-blocking anti-E- cadherin antibody, did not affect adhesion of the E-cad-expressing cells either to each other or to the P-cad-expressing cells (Fig. 3B). This demonstrates that the "heterotypic" cell aggregation seen here does indeed result from heterophilic adhesive bonds established between E- and P-cadherin protein molecules.

B-cad and N-cad

In the absence of inhibitory antibodies, N-cad- and B-cad-expressing L cells formed mixed aggregates in the laminar flow device at 6 rpm (Fig. 2D). Under the same conditions, inhibitory antibodies directed against B-cad substantially discouraged both the aggregation of B-cad-expressing cells (green) and their adhesion to cells expressing N-cad (Fig. 3C). Similarly, inhibitory antibodies directed against N-cad substantially discouraged both the aggregation of N-cad-expressing cells (red) and their adhesion to cells expressing B-cad (Fig. 3D). Inhibitory antibodies directed against either one of the cadherins failed to inhibit aggregation of the cells expressing the other cadherin. The addition of both inhibitory antibodies to the culture medium strongly suppressed all aggregation (Fig. 3E). These experiments demonstrate that, like E-cad and P-cad, N-cad and B-cad expressed on different cells can cross-react to form adhesive bonds.

LCAM and N-cad

Equivalent results were obtained in combinations of N-cad- and LCAM-expressing cells cocultured in the laminar flow device at 18 rpm for 90 min. In the absence of antibodies, the two cell populations coaggregated indiscriminately. Fab fragments of an N-cad-specific inhibitory monoclonal antibody (6B3) allowed the initiation of adhesions between the LCAM-expressing cells, but strongly discouraged the initiation of adhesions of the N-cad-expressing cells both to each other and to the cells expressing LCAM.



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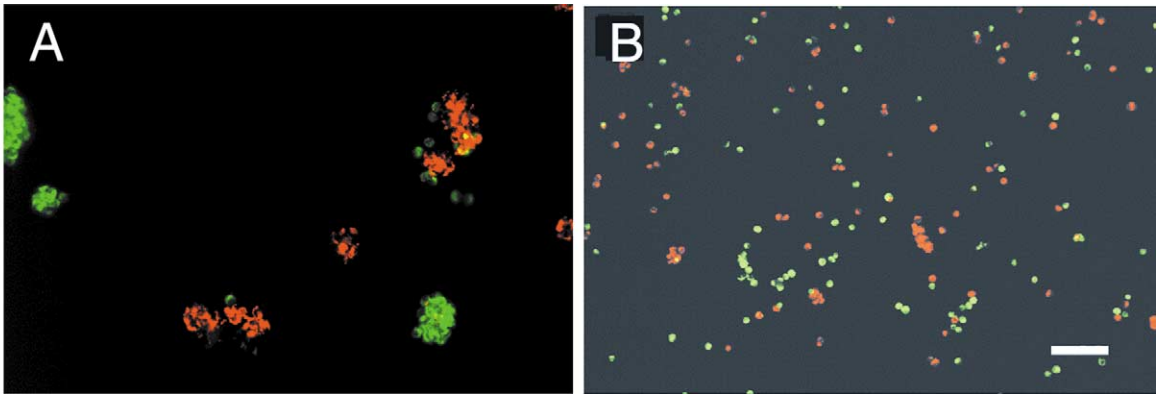

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Fig. 3. Inhibition of heterocadherin-mediated adhesion by inhibitory antibodies and Fab fragments directed against either cadherin. In a mixture of L cells expressing P-cad (red) vs E-cad (green) rotated in the laminar flow device for 100 min at 15 rpm, ECCD-1, a function- blocking anti-E-cad antibody, restrains both the homotypic and heterotypic aggregation of E-cad- expressing cells while permitting aggregation of cells expressing P-cad (A). ECCD-2, a non-function-blocking, anti-E-cadherin antibody did not affect aggregation (B). (C–E) B-cad-and N-cad- expressing cells and conditions are as in Fig. 2D. Addition of anti-B-cad-specific antibody 5A6 inhibits B-cad-mediated adhesions; principally homotypic adhesions between N2 cells (red) are formed (C). Addition of N-cad-specific antibody 6B3 restrains formation of N-cad-mediated adhesions; homotypic adhesions between B-cad-expressing cells (green) are favored (D). Addition of anti-B-cad and anti-N-cad antibodies inhibits the formation of all adhesions (E). Confocal images. Scale bar represents 100 μm.

Similarly, Fab fragments of an inhibitory rabbit polyclonal antibody against LCAM allowed the formation of adhesions between N-cad-expressing cells while restraining the formation of adhesions of LCAM-expressing cells both to each other and to cells expressing N-cad. When both antibodies were used together under the same conditions, formation of all cell–cell adhesions was suppressed (data not shown). Thus, in all cases examined, adhesion between L cells expressing different cadherins resulted from the ability of those cadherins to form adhesive bonds cross-linking the cells that display them.

Effects of shear upon the formation of heterocadherin adhesions

How can the published accounts of separate aggregation of cells expressing different classical cadherins be explained? We long ago pointed out the invalidity of using sheared cell suspensions to assess the ability of cells to initiate adhesions (Moyer and Steinberg, 1976; Steinberg, 1970). Since the shearing of a cell suspension both brings cells together and tears them apart, with stronger shear forces favoring cell separation, we have examined the role of shear forces in the appearance of homophilic selectivity of cadherin–cadherin intercellular adhesions. The laminar flow roller tube device was used below to produce moderate shear forces, while the gyratory shaker was employed to produce turbulent rheological conditions that expose cells suspended in our 10-ml culture flasks to high shear forces. The cadherin combinations described below illustrate the range of results we have obtained.

R-cad and B-cad

Although cells exclusively expressing R-cad vs B-cad coaggregated in the laminar flow device at 1.2 rpm (Fig. 2A), at moderately greater shear rates, a quantitative preference for “homocadherin” adhesion emerged. When the rotation rate was increased still further to 30 rpm, formation of heterocadherin adhesions was largely suppressed, each aggregate consisting mainly of cells of one or the other kind (Fig. 4A). These cells also formed separate aggregates in gyratory shaker cultures.

Our use of a laminar flow device in the above experiment permits a rough calculation of the duration of cell–cell contact required between our B-cad- and R-cad-expressing cells to permit stable adhesions to form in the shear gradients utilized. A spherical particle in a shear gradient moves, in a rolling motion, with the velocity of the medium at its center. The centers of two such particles coming into tangential contact are separated by a distance equal to the sum of their radii. Because these suspended cells extend noticeable protrusions, we estimate that contacts between them might occur as the faster moving cell traverses a distance of about three cell diameters relative to the slower moving cell. Knowledge of their radii and the shear gradient permits estimation of their relative velocity as one brushes past the other. In this device rotating at 30 rpm, two cells 10 μm in diameter would come into tangential contact at a relative velocity of about 167 μm/sec. If they remain in contact as the faster moving cell traverses a distance of 30 μm relative to the slower moving cell, then they would do so for about 180 ms. Under these conditions, many “homotypic” (B-cad to B-cad; R-cad to R-cad) but few “heterotypic” (B-cad to R-cad) adhesions survived (Fig. 4A). At 1.2 rpm, two colliding cells would remain in mutual contact for about 4.5 s. Under these conditions, “heterotypic” adhesions were about as abundant as “homotypic” ones (Fig. 2A). It may be concluded that, under the conditions utilized, a sufficient number of trans “homocadherin” interactions between either B-cad or R-cad molecules expressed on the contacting cell surfaces can be formed in about one-fifth of a second to resist reseparation by the shear forces that first bring the cells into contact and then act to separate them. Formation of a sufficient number of “heterocadherin” interactions between B-cad and R-cad molecules expressed on the contacting cell surfaces requires longer contact, estimated as not exceeding about 4.5 s, and/or lower shear forces.

LCAM and N-cad

Whereas cells expressing exclusively LCAM vs N-cad coaggregated in the laminar flow device at 3.3 rpm (Fig. 2B), at shear forces an order of magnitude higher, a preference for homocadherin adhesion was seen. At 33 rpm, aggregation of all cells in this mixture was greatly discouraged, but the few cell associations that persisted after overnight culture were preferentially between cells expressing the same cadherin (Fig. 4B).

P-cad and E-cad

In the above two combinations, cells expressing either the same or different cadherin subtypes coaggregated under lower shear conditions. Increasing the shear forces above a critical level selectively discouraged the formation of adhesions between cells expressing different cadherins, giving a misleading appearance of homocadherin binding specificity. In the combination of these two L cell lines, coaggregation of cells expressing E-cad and P-cad was not prevented even at 120 gyres/min on the gyratory shaker—the highest shear conditions attainable without frothing the medium. As already noted, this is the same pairing of cadherins earlier found (Nose et al., 1988) to produce separate aggregates in stirred suspensions of transfected L cells.

Fig. 4. Selective suppression of heterocadherin-mediated adhesion by elevated shear forces. L cells expressing B-cad (green) vs R-cad (red) formed largely separate aggregates in the laminar flow device during 90 min at 30 rpm (A). L cells expressing LCAM (green) vs N-cad (red) aggregated very slowly during overnight rotation in the laminar flow device at 33 rpm, with a preference for self-adhesion (B). Confocal images. Scale bar represents 100 μm.

Table 1
Expression levels of E-, P- and N-cadherin on surfaces of transfected L cell lines

Cell line	Cadherin expressed	Surface cadherins per cell ($\times 10^4$)
E8a	E	2.37
LE-Dex (uninduced)	E	3.51
LP1	P	3.55
N2	N	4.75
N5A	N	7.23
LE-Dex (+1 μM dex)	E	~15.8

Effects of shear upon the formation of homocadherin adhesions

Higher vs lower cadherin expression

The above experiments demonstrate the ability of shear forces to selectively discourage the formation of adhesions between cells expressing different cadherins, giving the misleading impression that these cadherins are unable to form cross-bridges. However, we have also observed examples of separate aggregation in gyratory shaker cultures of paired cell populations expressing the identical cadherin at different levels. Using L cell lines transfected to express N-cadherin, we selected two clones expressing N-cad in moderately different ranges. The expression ranges of these two cell lines overlap, but line N5A displays a peak surface N-cad expression level of about 7.23×10^4 cadherins per cell, about 50% higher than cell line N2, with a peak surface N-cad expression level of about 4.75×10^4 cadherins per cell (Table 1). When these two cell lines are allowed to aggregate in separate vessels under the same conditions, the ~50% difference in mean cadherin expression level causes the N5A cells to aggregate faster than the N2 cells, forming larger aggregates in a shorter time.

These two cell lines were dissociated with trypsin–EDTA (TE-treatment) and fluorescence-labeled red vs green. When 3-ml aliquots of a 1:1 mixture were cultured overnight at 37°C in 10-ml gyratory shaker flasks under high-shear conditions (120 gyres/min), aggregates of two kinds formed. The majority of aggregates contained both red-labeled (N2) and green-labeled (N5A) cells, but a subset of aggregates contained only green-labeled (N5A) cells (Fig. 5). No aggregates consisted solely of the lower-expressing N2 cells. The difference in intrinsic aggregation rates of differentially labeled cells displaying different numbers of identical cadherin molecules is sufficient to allow the formation of some aggregates containing exclusively the most rapidly aggregating N5A cells (green), presumably those with the highest cadherin expression levels, presenting an impression of qualitative adhesive specificity where none exists. In this circumstance, the formation of aggregates containing only lower-expression cells is not to be expected because they can form at least as many bonds with higher-expression cells as they can with each other, and the bonds themselves are all the same.

Cell sorting within mixed aggregates

The preceding experiments have examined the conditions that cause separate aggregation of differing cells within sheared, heterogeneous cell suspensions. We refer to this as “separate aggregation” to distinguish it from the entirely distinct phenomenon of demixing or sorting-out of different cell combinations already cohering within individual aggregates. The latter phenomenon reflects not any lack of mutual adhesiveness but rather the immiscibility of cell populations that are, nevertheless, mutually adhesive. It has historically been called “sorting-out” or “cell sorting” (Moscona and Moscona, 1952; Steinberg, 1962a–c; Townes and Holtfreter, 1955), a term that, to avoid confusion, ought not to be used to describe “separate aggregation” (e.g., Niessen and Gumbiner, 2002).

P-cad- and E-cad-expressing cells

For these experiments, we used the P-cad-expressing L cell line LP1 and two E-cad-expressing L cell lines. These were LE-Dex, expressing E-cad under the control of a dexamethasone-inducible promoter, and E8a. The absolute mean surface expression levels of their respective cadherins by E8a, LP1, and uninduced LE-Dex cells were all determined by flow cytometric comparison of cell surface immunofluorescence with that of a series of QSC microspheres of known antibody-binding capacity (Brockhoff et al., 1994; Zagursky et al., 1995). Overnight culture of LE-Dex cells in medium containing 1 μM dexamethasone increased their mean E-cad expression level about 4.5-fold, as determined by direct flow immunocytometric comparison. LE-Dex cells have a basal level of E-cad expression that is very close to the P-cad expression level of LP1, E8a cells having a lower and induced LE-Dex cells a higher surface cadherin expression level (Table 1).

P-cad-transfected LP1 cells, expressing about 50% more cadherin than E-cad-transfected E8a cells, sorted out with the former completely enveloped by the latter (Fig. 6A). The same LP1 cells, expressing less cadherin than the E-cad-transfected LE-Dex cells (+1 μM dex) sorted out in the reverse configuration, with the latter completely enveloped by the former (Fig. 6C). Both results are consistent with the principle of the Differential Adhesion Hypothesis that more cohesive cell populations segregate internally to less cohesive ones to which they adhere, regardless of the identities of the molecules mediating those adhesions. When P-cad and E-cad expression levels were equalized, however [LP1 vs LE-Dex (uninduced)], the two cell lines did not sort out but remained intermixed (Fig. 6B). Two conclusions follow. First, E-cad and P-cad must produce about equal adhesiveness on a molar basis, a conclusion supported by direct measurements of cell aggregate surface tensions (to be re-

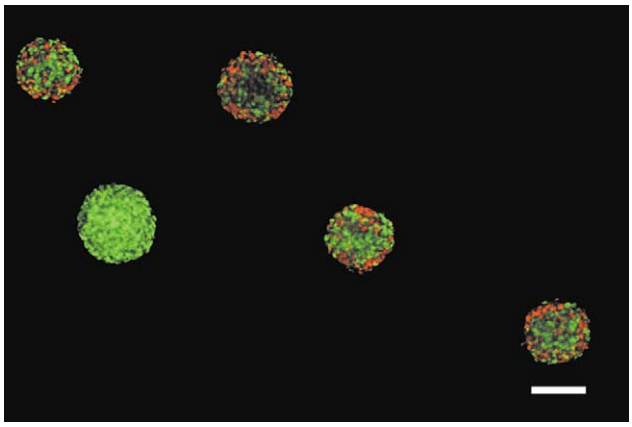

Fig. 5. Some cells expressing higher levels of a given cadherin form separate aggregates under high-shear conditions. Low N-cad-expressing N2 cells are labeled red, higher N-cad- expressing N5A cells are labeled green. A mixed single-cell suspension of the two cell lines was allowed to aggregate overnight at 120 gyres/min on a gyratory incubator-shaker. Some of the higher-expression cells formed separate aggregates. Confocal image. Scale bar represents 100 μm.

ported separately). Moreover, these two cadherins must also cross-adhere with an affinity similar to that with which each self-adheres. If their "cross-affinity" was significantly weaker than their "self-affinity," cells expressing them in equal numbers should segregate within a common aggregate (Steinberg, 1962, 1963, 1964, 1978). The sorting-out of L cell populations uniquely expressing these two cadherins is therefore largely or entirely due to quantitative differences in their expression and not to significant differences in binding affinity between these two cadherin subtypes. These results were reported earlier in abstract form (Duguay and Steinberg, 1999).

Moderate differences in cadherin expression level suffice to cause cell sorting

A major difference (about 20-fold) in P-cad expression level between paired L-cell populations was earlier shown to produce cell sorting, tissue spreading, and specific tissue layering (Steinberg and Takeichi, 1994). Here, we have asked how small a difference in cadherin levels is sufficient to produce these morphogenetic consequences. We utilized two L-cell lines expressing N-cad at moderately different levels. Line N5A expresses about 50% more N-cad than does line N2 (Table 1). Cells of these two lines were stained to fluoresce red or green, mixed in equal numbers, and pelleted. The thin pellets were cut into small fragments, which were placed in gyrating culture. Fig. 7A is a confocal section through the center of such an aggregate cultured for a day, in which both the red- and the green-labeled cells are from the N5A line and therefore express N-cad at the same level. The initially flat and approximately square aggregates had rounded up to become spheroids, indicating that the cells were capable of rearranging, but the two cell populations did not segregate. (Coaggregated red-and green-stained cells of the same kind remain intermixed in all cell lines examined; our unpublished data.) Fig. 7B is a confocal section through the center of a similarly prepared aggregate containing a mixture of N5A and N2 cells. The 50% difference in mean N-cad expression level between these two lines was sufficient to cause them to segregate from one another during 1 day of culture, with the lower-expressing N2 (green) cells completely enveloping the higher-expressing N5A (red) cells. As before, lower cohesion causes external positioning. In preliminary experiments, even a 26% difference in mean E-cad expression level has been sufficient to produce a degree of cell sorting in mixed

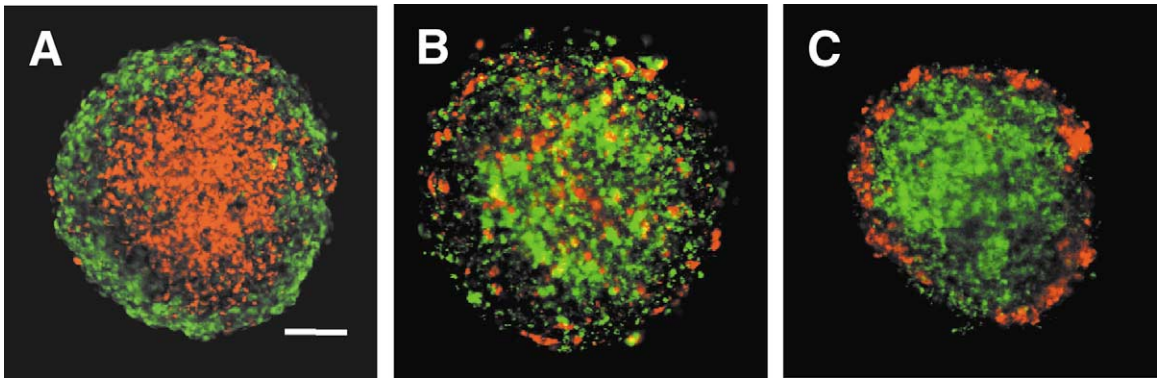

Fig. 6. L cells expressing E- vs P-cad sort out only when they differ in cadherin expression level. The E-cad expression level of dexamethasone-inducible L cell line LE-Dex was controlled by adjusting the concentration of dexamethasone in the medium. Due to the leakiness of the promoter, in the absence of dexamethasone, the mean expression level of this line (3.51×10^4 cadherins per cell) was similar to that of P-cad-expressing line LP1 (3.55×10^4 cadherins per cell). When 1 μM dexamethasone was added to the culture medium overnight, the mean E-cad expression of LE-Dex cells was increased about 4.5-fold to $\sim 15.8 \times 10^4$ cadherins per cell. L cells expressing P-cad (red) or E-cad (green) were copelleted and formed a coherent aggregate. This was then cut into small pieces that were cultured in suspension for 2 days. In (A), E-cad- expressing cell line E8a, paired with cell line LP1, expressing P-cad at a higher level (Table 1), segregated externally. In (B) and (C), inducible E-cad-expressing cell line LE-Dex was used. In (B), E-cad expression was approximated to that of the P-cad line and no sorting-out occurred. In (C), the E-cad-expressing line LE-Dex was induced to an expression level greater than that of the P-cad-expressing line LP1 and segregated internally. Confocal images. Scale bar represents 100 μm.

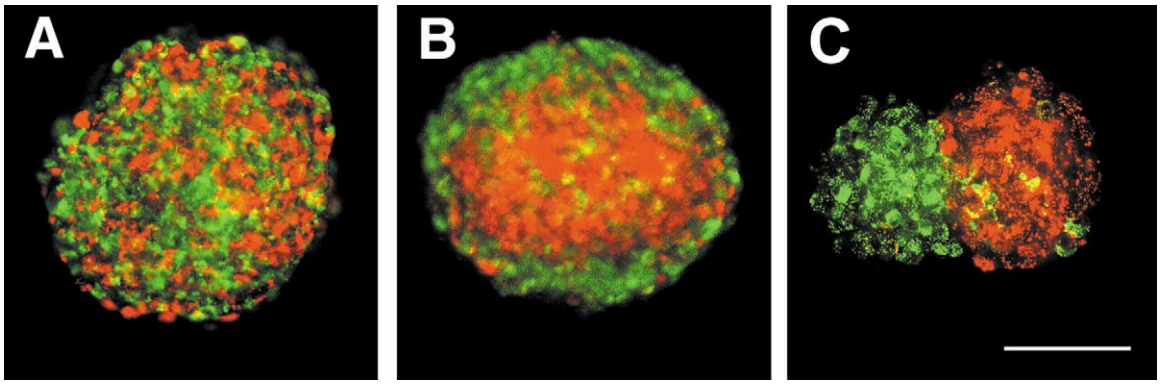

Fig. 7. Equilibrium configurations of L cell mixtures as a function of the number and kind of cadherins expressed. Equal numbers of cells were stained, intermixed, and pelleted by centrifugation, then cut into fragments and cultured in suspension for 1 (A, B) or 2 days (C) to allow the aggregates to reorganize. (A) An aggregate containing a mixture of red- and green-labeled N5A cells rounded up, the identical cells remaining intermixed. (B) N5A cells (red), expressing a 50% higher level of N-cad, segregated internally to N2 cells (green) expressing the same cadherin at a lower level. (C) Aggregates containing equal numbers of L cells expressing B-cad (green) and R-cad (red) segregated to produce mounds of R-cad-expressing cells partially capping a B-cad-expressing mass. Confocal images. Scale bar represents 100 μm.

aggregates cultured for two days (A. Flagg, undergraduate thesis).

Equilibrium configurations of paired cell populations provide criteria of adhesive selectivity

The results presented above and discussed below demonstrate that neither of the two criteria (separate aggregation of two cell populations in a sheared suspension or cell sorting within a common aggregate) used in the past as evidence of type-specificity of cadherin-mediated cell–cell adhesion is valid. A valid criterion does, however, exist. Fig. 7B shows the final configuration arrived at by the self-organization of paired cell populations expressing the same cadherin in different amounts. This configuration is one of complete envelopment of the higher-expression cell population by its lower- expression partner and represents the outcome mathematically predicted (Steinberg, 1962c, 1963, 1964) and empirically found (Steinberg and Takeichi, 1994) in the absence of affinity differences between the two cell populations' adhesion sites. Sorting-out of two cell populations to produce this configuration of complete envelopment of one cell population by another therefore offers no evidence of selective disaffinity between the differing cells' adhesive sites, e.g., E-cad and P-cad as seen in Fig. 6. When coaggregates of B-cad- and R-cad-expressing L cells were cultured for 2 days, however, they self-organized to form a different configuration. These two cell populations segregated within each aggregate to produce not a configuration of complete envelopment of one cell population by the other, but rather one or more lumpy masses of red fluorescent R-cad cells perched as caps partially enveloping a mass of green fluorescent B-cad cells (Fig. 7C).

In cell populations that display liquid-like behavior (cell sorting, rounding-up of suspended cell aggregates, the spreading of one cell aggregate over the surface of another), as in ordinary systems of immiscible liquids, incomplete envelopment of one phase by the other at configurational equilibrium theoretically signifies that the cross-adhesion between the two phases is weaker than the self-adhesion of either phase (Steinberg, 1962c, 1963, 1964, 1978). In cellular systems, this circumstance would require a lesser affinity between the differing cell populations' adhesive sites (in this case, B-cad and R-cad) than can be accounted for by quantitative differences in their expression levels alone. B-cad and R-cad expressed on L cells can cross-adhere, but the incomplete envelopment of B-cad-expressing by R-cad-expressing L cells at configurational equilibrium implies that mature adhesions between these two cadherins must be weaker on a molar basis than those between paired cadherins of either kind.

Discussion

Cross-adhesion between different cadherins

Initial reports and many secondary accounts have represented cadherin-mediated cell–cell adhesion as being more or less cadherin subtype-specific (Inuzuka et al., 1991; Miyatani et al., 1989; Munchberg et al., 1997; Murphy-Erdosh et al., 1995; Nose et al., 1988; Takeichi et al., 1985). However, cells expressing different classical cadherins have been shown to be capable of cadherin-mediated mutual adhesion in a number of cases. Volk et al. (1987) reported that cocultured chick lens cells, displaying N-cad, and chick liver cells, displaying LCAM, form adherens-type junctions displaying these two adhesion molecules in apposition. The formation of these chimeric junctions could be inhibited by antibodies directed against either molecule, leading the investigators to propose that the two cadherins interact directly, a conclusion that we here confirm. Cross-adhesion

has been reported between N-cad and the closely related R-cad, expressed individually on transfected L cells (Inuzuka et al., 1991; Matsunami et al., 1993); between B-cad and both LCAM and E-cad and between LCAM and E-cad (Murphy-Erdosh et al., 1995). We have previously reported the cross-adhesion of cells expressing E- and P-cad (Duguay and Steinberg, 1999). Interactions between different cadherins expressed on epitheliocytes and fibroblasts have recently been described (Omelchenko et al., 2001) as have interactions among a number of type II classical cadherins (Shimoyama et al., 2000). CHO cells expressing human N-cad, human or Xenopus E-cad, or Xenopus C-cad have recently been found to bind indiscriminately to purified and immobilized dimerized extracellular domains of human E-cad or Xenopus C-cad (Niessen and Gumbiner, 2002). Here, we present further evidence of heterocadherin adhesion among type I cadherins and identify the experimental conditions that have previously masked the detection of this cross-adhesion.

Shear-dependent selectivity of cell aggregation

The concepts of "homotypic" specificity of adhesion between cells of different kinds and later of "homophilic" subtype-specificity of adhesion between cadherins have both been based in significant measure upon a highly artificial assay procedure: observations of differences in the *rates* of adhesion between like and differing cells in sheared suspensions (Murphy-Erdosh et al., 1995; Nose et al., 1988; Roth, 1968; Roth and Weston, 1967; Takeichi et al., 1981). Shear forces, however, both bring cells into contact and pull them apart. It is axiomatic in chemical physics that forward reaction rates do not measure affinities (Glasstone et al., 1941). This applies as well to the kinetics of adhesion formation between cells in sheared suspensions, in which there does not appear to be a significant off-rate, as it does to the forward reaction rates of other chemical reactions in solution (Steinberg, 1964, 1970). This is true not only because binding energies cannot be deduced from forward binding rates but also because cadherin-mediated adhesions between cells are greatly strengthened by interactions among and between cadherins and other cytoplasmic proteins during the course of an hour or so subsequent to initial cell–cell contact (Adams et al., 1996; Angres et al., 1996; Brieher et al., 1996; Ozawa and Kemler, 1998). Aggregation rates cannot reveal the properties of these physiologically relevant adhesions, whose enormous strengthening does not occur until after initial adhesions have taken place.

Previous studies of the ability of L cells expressing different classical cadherin subtypes to cross- adhere in sheared cell suspensions have employed a fixed shear rate (Murphy-Erdosh et al., 1995; Nose et al., 1988). A range of behaviors were observed. In some combinations, the two cell populations coaggregated indiscriminately. In one combination, the two populations coaggregated, but to a limited degree. In several combinations, the two populations aggregated separately at first, but these separate aggregates subsequently cross-adhered. In still other combinations, the two populations formed separate aggregates that were not reported to cross-adhere with longer coculture. The role played by the shear forces operating to produce these various results was not explored.

Shear forces produce the analog of an activation energy barrier to aggregation (Steinberg, 1970), which can be raised or lowered by adjusting the shear rate. Thus, stirring a heterogeneous cell suspension can permit the formation of certain rapidly formed adhesions while preventing the formation of other adhesions that form more slowly. A failure to form "heterotypic" aggregates in such a circumstance provides no evidence concerning the ability of the differing cells to adhere, even strongly, in the absence of shearing. This is shown here in a mixture of two L cell populations expressing surface N-cad molecules in the ratio of about 3:2, allowed to coaggregate on a gyratory shaker. While many aggregates incorporated cells expressing both more and less N-cadherin, some of the more highly expressing cells formed aggregates containing few if any of the lower-expression cells (Fig. 5). "Selective cell adhesion" in this case results not from any differences in molecular specificity of the adhesion molecules but from the ability of cells possessing more binding sites to adhere more rapidly in the presence of shear forces. Other demonstrations here of misleading impressions of adhesive specificity due to the imposition of shear forces include the coaggregation of L cells expressing N-cad vs LCAM and B-cad vs R-cad at lower shear forces but their formation of separate aggregates at higher shear forces (Figs. 2 and 4). It has been reported that L cells expressing E-cad vs P-cad initially failed to cross-adhere when mixed in cell suspensions on a gyratory shaker (Nose et al., 1988). In the present experiments, L cells expressing these same two cadherins were observed to co-aggregate readily (Fig. 2), even at high shear forces. (These different results are consistent with the possibility that the transfected cells used in the earlier experiments expressed E-cad and P-cad at significantly lower levels than ours. The initiation of cross-adhesions between such lower-expressing cells would be more readily disrupted by shear forces.) These same cells, in our experiments, also failed entirely to sort out when the two cadherins were expressed at the same level (Fig. 6B), indicating that the mature "heterocadherin"-mediated adhesions in this case, far from being very weak (Nose et al., 1988), are similar in strength to the two related kinds of mature "homocadherin"-mediated adhesions.

We have noted here that different levels of shear differentially prevent the initiation of adhesions between cells expressing various cadherins at various levels. In extensive experiments to be reported elsewhere, we have found no case in which L cells expressing different classical cadherins have failed to cross-adhere, in most cases quite strongly, in the absence of shear (unpublished data).

The effects of stirring upon the collisions of suspended cells or other particles have been treated formally by Smolu-

chowski (1916, 1917). In relating these calculations to cell aggregation kinetics, we have pointed out that "the probability of collision" (of a reference particle with another particle) "increases with the third power of the radius" (Steinberg and Roth, 1964). The effect of this is to cause already-formed aggregates to collide with others at a greatly increasing rate, promoting the further fusion of already-formed aggregates, as is shown in Table 1 in Steinberg et al. (1973). This may account for the secondary fusion between earlier-formed "homotypic" aggregates described by Nose et al. (1988). In short, the various effects of shear forces in stirred suspensions of cells which, while mutually adhesive, form cross-adhesions at different rates, can account for much behavior that has previously been interpreted as indicating cadherin subtype-specificity of adhesion.

What is the biological significance of the fact that shearing rates can be found that prevent the initiation of adhesions between cells expressing two different cadherins but permit the initiation of adhesions between cells expressing either one of these cadherins? Our calculations indicate that sufficient "homocadherin"—but not "heterocadherin"—bonds between our cell lines expressing B-cad or R-cad can be formed within about 180 ms to resist separation by the shear forces produced in our laminar flow device rotating at 30 rpm. By contrast, it takes as much as 25 times as long for sufficient heterocadherin bonds to form between these cells to resist separation by shear forces 1/25th as great. We interpret these findings to mean that when cadherins on apposed cells first come into contact, identical cadherin cis dimers on the two cells' surfaces can, in at least some cases, align themselves in the positions required for trans dimerization (Baumgartner et al., 2000; Brieher et al., 1996; Chappuis-Flament et al., 2001; Chitaev and Troyanovsky, 1998; Pertz et al., 1999) more rapidly that can nonidentical cadherin cis dimers. Since (1) these alignments occur within a few seconds in either case and (2) motile cells encountering one another in their natural circumstances remain in contact for much longer time periods and are not subjected to shear forces, such kinetic differences in intermolecular association rates seem unlikely to serve as a basis for the cellular associative choices that bring about tissue segregation and guide tissue organization in normal morphogenesis. Recent crystallographic studies of C-cad structure (Boggon et al., 2002) have identified conserved elements in the structure of classical cadherins that provide a plausible structural basis for their promiscuous mutual binding.

After cadherin-mediated cell–cell adhesions are initiated, they become progressively strengthened during the next hour or so by a process involving multimerization and the formation of a complex including catenins and actin (Adams et al., 1996; Angres et al., 1996; Baumgartner et al., 2000; Brieher et al., 1996; Chitaev and Troyanovsky, 1998). Our evidence indicates that it is the relative strengths of these mature intercellular adhesions, measurable as cell aggregate surface tensions, that specify whether motile cell populations will intermix or segregate and the anatomical configurations they will tend to adopt (Davis et al., 1997; Foty et al., 1994, 1996; reviewed in Steinberg, 1996).

Sorting-out of cells does not imply molecular disaffinity

In addition to the separate aggregation of differing cells in mixed, sheared suspensions, a second behavior of heterogeneous cell populations widely invoked as evidence of adhesive *disaffinity* between cells is the sorting-out of cells within such mixtures. This also is a misconception, however, as shown by our demonstrations that two cell populations identical except in the expression level of a given cadherin sort out or spread, one over the surface of the other, to form a sphere-within-a-sphere configuration in which the higher expression cells are segregated from and totally enveloped by the lower expression cells (Steinberg and Takeichi, 1994; and Fig. 7B). Even a moderate difference in cadherin expression level (3:2 in Fig. 7B) is sufficient to cause cell sorting. Therefore, the phenomenon of sorting-out of cells expressing different cadherin subtypes within a common aggregate offers no evidence of differential affinity of the cadherin subtypes unless these are expressed in equal amounts. None of the many observations that embryonic tissue boundaries coincide with domains of differential cadherin expression (e.g., Redies, 2000; Takeichi, 1988) have been accompanied by accurate measurements of the expression levels or true binding affinities of the cadherins in these adjoining domains.

Evidence of intermolecular adhesive selectivity from equilibrium configurations of paired cell populations

Fig. 7B illustrates the complete envelopment of a more cohesive by a less cohesive cell population that takes place in the absence of any difference in the affinities of the adhesion molecules (all N-cadherin in this case) linking the cells. Another example, using P-cadherin-expressing cells, is shown in Fig. 3 in Steinberg and Takeichi (1994). Emergence of this configuration in combinations of cells expressing non-identical or unknown adhesion molecules provides a clear indication that the cross-adhesions between the segregating heterotypic cells are about as strong as if their adhesion molecules were identical. Observation of this configuration in combinations of L cells expressing different amounts of E- vs P-cadherin (Figs. 6A and 6C) leads to the conclusion that there is little or no difference between the self- and cross-affinities of those two cadherins. That conclusion is bolstered by the observation that L cells expressing E- vs P-cadherin in equivalent amounts do not sort out (Fig. 6B). Interestingly, complete envelopment is the final configuration observed in the great majority of experimental combinations of chick embryonic cells and tissues, expressing a variety of cadherins (e.g., Steinberg, 1970). In certain cell combinations, however (36-h posterior neural tube with either 5-day pigmented epithelium or 5-day liver), the equilibrium configuration was one of partial rather than com-

plete envelopment (Steinberg, 1970), suggesting a significantly lower affinity between adhesion molecules expressed by those particular differing cells. This was also the case observed for combinations of B-cad- and R-cad-expressing L cells in the present experiments (Fig. 7C).

Unlike the aggregation behavior of cells in sheared suspensions or the sorting-out of intermixed cells within coherent cell aggregates, the equilibrium configurations approached by heterogeneous combinations of cells uniquely expressing defined adhesion molecules in precisely measured amounts can be used to determine the true relative affinities among cadherins and other adhesion molecules in fully developed associations. The configurations produced by combinations of L cells expressing other cadherins have been examined and will be reported subsequently. We believe that the more general adoption of these criteria has much to contribute to the clarification of the physical and molecular bases of adhesion-based morphogenetic phenomena.

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